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RAPID COMMUNICATION

REDUCTION OF DIHYDROXYPHENYLACETIC ACID BY A NOVEL ENZYME IN THE RAT BRAIN

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ABSTRACT - A novel enzyme that converts dihydroxyphenylacetic acid (DOPAC) to dihydroxyphenylethanol (DOPET) was found to be present in the microdialysate of the rat brain. The enzyme, named DOPAC reductase, was inhibited by EDTA and stimulated by divalent cations like Zn^{2+}, Mn^{2+}, Co^{2+} and Cu^{2+}. Its K_m, pH optimum and temperature optimum were found to be 32 ± 2 μM, 7.5 and 40°, respectively. The equivalent acid metabolite of noradrenaline, 3,4-dihydroxymandelic acid, and the methoxylated acids of both noradrenaline and dopamine, 3-methoxy-4-hydroxymandelic acid and 3-methoxy-4-hydroxyphenylacetic acid, were found not to be substrates of DOPAC reductase. Thus, DOPAC reductase may be an enzyme that is specifically involved in the one-step conversion of DOPAC to DOPET in the central metabolism of dopamine.

Key words: DOPAC reductase; DOPET; microdialysis; rat brain

Recently, we described a novel soluble form of MAO[+] that converts dopamine to DOPAC in the microdialysate of rat brain [1]. In the ensuing kinetic study of the enzyme, we found that DOPAC formed in the incubation mixture was converted simultaneously to another product. This hampered the kinetic study on the MAO in the microdialysate and prompted us to study the conversion of DOPAC to the new product. The present communication reports the existence of a new enzyme that converts DOPAC to DOPET and discusses the likely role of the enzyme in the central metabolism of dopamine. For ease of mention, the new enzyme is referred to as DOPAC reductase.

MATERIALS AND METHODS

The implantation of the microdialysis probe into the arcuate nucleus/median eminence of each anaesthetized animal (pentobarbital sodium 5 mg/100 g, i.p.) was carried out as described previously [1]. The microdialysis probe was made from 40,000 Da cutoff Hospal dialysing fiber (Hospal Ltd., U.K.) of about 0.22 mm outer diameter and vitreous silica tubing (Scientific Glass Engineering, U.K.) of 0.14 mm outer diameter; 40,000 Da cutoff dialysing fiber was chosen because lower molecular weight cutoff fiber, e.g. 20,000 Da cutoff, yielded poor recovery of the enzyme of interest. The effective dialysing length was

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+Abbreviations: MAO, monoamine oxidase; DOPAC, dihydroxyphenylacetic acid; DOPET, dihydroxyphenylethanol; DOMA, 3,4-dihydroxymandelic acid; VMA, 3-methoxy-4-hydroxymandelic acid; HVA, 3-methoxy-4-hydroxyphenylacetic acid; and CSF, cerebrospinal fluid.

1 mm, and the average recovery for DOPAC was 15 ± 3%. The coordinates of implantation with reference to the bregma were A-P 1.8 mm, M-L 0.25 mm; depth from skull surface = 10.35 mm. The probe was implanted for chronic microdialysing, and it was held in place by dental cement.

On the day following implantation, the rat was connected to a swivel system for a freely moving animal (CMA/120, Carnegie Medicin), and artificial cerebrospinal fluid was perfused through the probe at a rate of 1 µL/min using a microinjector (CMA 100, Carnegie Medicin). After an hour of acclimatization, 20-µL samples were collected for the assay of the new enzyme.

The enzyme assay was initiated by adding 5 µL of dialysate to an incubation mixture containing 30 µM DOPAC and 50 µM phosphate buffer, pH 7.4, in a total volume of 20 µL. Aliquots of 5 µL artificial cerebrospinal fluid instead of dialysate were added to the same samples of incubation mixture to obtain the control for each assay. The enzymatic reaction was terminated at various time intervals by the addition of 5 µL of 3 M perchloric acid. The controls were similarly treated with perchloric acid. The product, DOPET (see Results for its identification by mass spectrometry), was separated from DOPAC by high performance liquid chromatography and quantitated by electrochemical detection. Briefly, the chromatography system consisted of a Waters 510 solvent delivery pump, a Rheodyne 7125

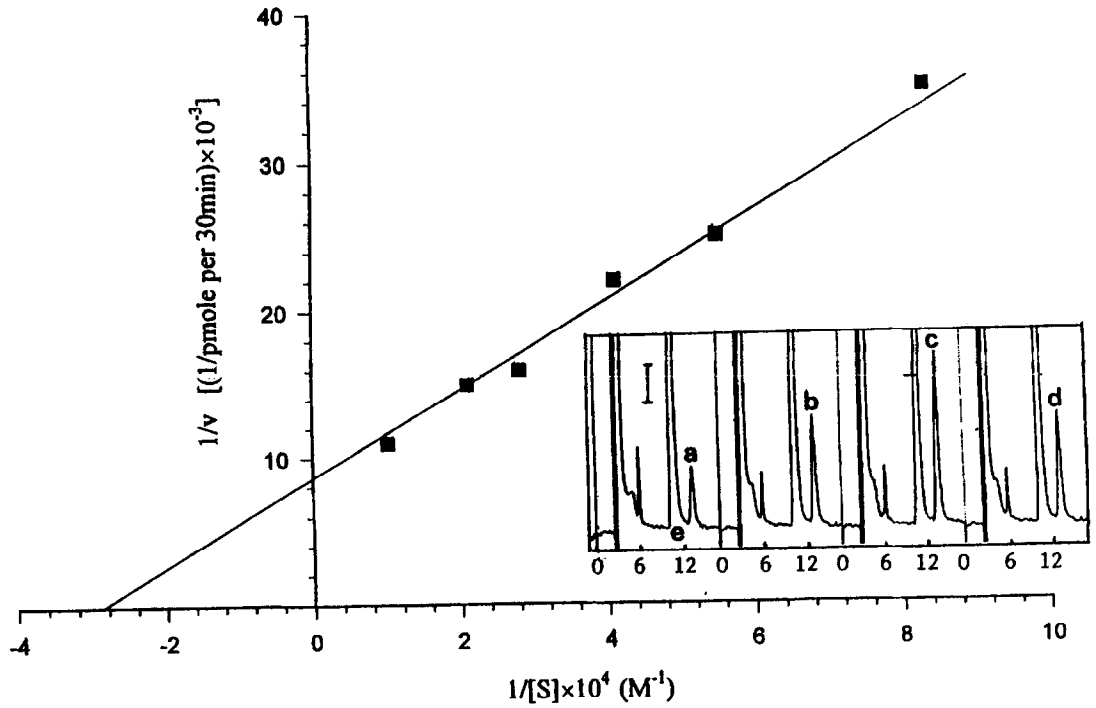

Fig. 1. Double-reciprocal plot of the enzymatic formation of DOPET from DOPAC by DOPAC reductase. Each point is the mean of a duplicate assay. Each assay was carried out by incubating the enzyme with a different substrate concentration (12-96 µM) for a period of 30 min. Inset: Chromatogram of DOPET at various times of enzyme incubation (see text for details). Peaks a, b and c are DOPET peaks obtained at 15, 30, and 45 min of enzyme incubation, respectively. Peak d was obtained at 15 min of incubation with twice the amount of dialysate. The off-scale peak (labelled e in the first chromatogram) in each chromatogram is the DOPAC peak. 0 indicates the time of injection and 6 and 12 the time in minutes after the injection. The bar represents 1 nA.

injector, a Merck 250 x 4 mm, 5-μm C_{18} column, and a Waters 464 Pulsed Electrochemical Detector. The mobile phase was a mixture of 75 mM potassium phosphate buffer, pH 5, containing 1 mM EDTA and 4% methanol. The flow was isocratic at a rate of 1 mL/min. Using the same protocol but with a mobile phase of different pHs, DOMA, VMA, and HVA were tested as likely substrates of DOPAC reductase.

RESULTS

The formation of DOPET in the first 45 min was linearly related to the time of incubation and proportion to the volume of dialysate added (see inset of Fig. 1). DOPET was not formed in control assays that were incubated for up to 60 min. These results, together with the fact that the organic synthesis of DOPET required 18 hr of reduction of DOPAC by lithium aluminum hydride, show that the formation of DOPET in the incubation mixture was enzyme catalysed. Figure 1 shows the double-reciprocal plot of the formation of DOPET. The K_m, which was determined using three different ranges of substrate concentrations (3–24, 6–48, 12–96 μM), was found to be 32 ± 2 μM. The V_{max} obtained from the corresponding three plots was 115 ± 7 pmol per 30 min per 20 μL dialysate.

Figure 2 shows the mass spectra of a sample of the enzymatically formed and of the custom-made (Tocris Cookson, U.K.) DOPET. DOPAC reductase was stimulated by Zn^{2+}, Mn^{2+}, Co^{2+} and Cu^{2+}; 1 μM of the four divalent cations enhanced its activity by 1.9-, 2.5-, 3-, and 5.8-fold, respectively. However, Mg^{2+}, Ca^{2+}, Fe^{2+} and Fe^{3+} had no effect on the enzyme

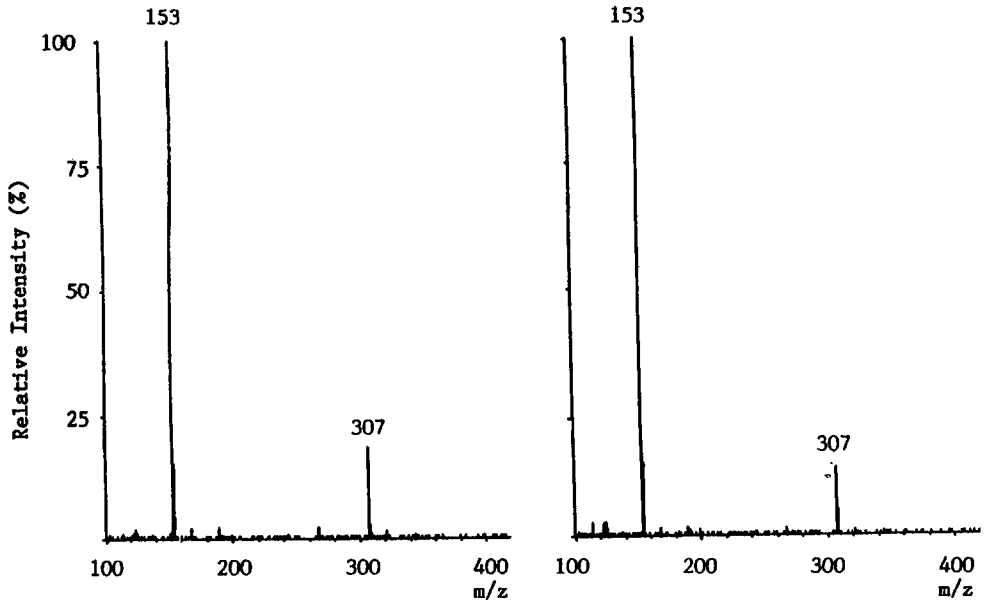

Fig. 2. Mass spectra of DOPET. The left spectrum pertains to that of custom-made DOPET and right spectrum to that of the enzymatically formed DOPET. One hundred samples of enzymatically formed and chromatographically separated DOPET were collected. The samples were pooled and lyophilized. The DOPET in the lyophilized powder was extracted with ethyl acetate and the ethyl acetate evaporated under vacuum to yield the DOPET. The mass spectrometry was performed on a Perkin Elmer SCIEX API III Plus LC/MS/MS system fitted with an ion spray interface. All analysis was done in the MS mode. Five microlitres of an aqueous filtered sample containing about 10 ng/μL was loaded into a 20-μL sample loop and injected into the mass spectrometer at a flow rate of 20 μL/min of 10% methanol using a Perkin Elmer series 200 pump. The ion peaks corresponding to a mass of 153 and 307, respectively, pertain to the mono and probably the dimer species of DOPET.

activity. EDTA inhibited the enzyme by 11, 29 and 100% at 1, 10 and 100 μM, respectively. The inhibition by EDTA could be reversed by addition of the four divalent cations that enhanced its activity, e.g. inhibition by 10 μM EDTA was reversed completely by 1 μM of the cations. The enzyme showed a pH profile with a pH optimum of around 7.5. When assayed at different temperatures for 20 min, it showed maximum activity at 40°. Activity deteriorated rapidly at a temperature above 60° and the enzyme lost its activity at 90°. DOPAC and DOPET in controls were stable at 90° during the 20 min of incubation. Incubation with 0.01 mg/mL trypsin at 37° for 45 min destroyed the enzyme activity. DOMA and the methoxylated acids of both noradrenaline and dopamine (VMA and HVA) were found not to be substrates of the enzyme.

DISCUSSION

DOPAC reductase and the isoenzyme of MAO [1] may form an important pathway for the metabolism of extracellular dopamine. Dopamine upon its release and activation of dopaminergic receptors can undergo oxidative deamination by extracellular MAO. The product, DOPAC, is then reduced to DOPET by DOPAC reductase. DOPET, being nonpolar, can either re-enter the terminals, nonneuronal cells, or be excreted from the brain. This pathway may account for the findings of earlier investigators, who demonstrated that, when concentrations of DOPAC and HVA are changed by drug treatment, only the concentration of HVA, and not that of DOPAC is increased by the administration of probenecid [2,3]. Similarly, inhibition of catechol-O-methyltransferase by tropolone following drug treatment, which accelerates the synthesis of HVA from endogenous dopamine, causes only a small increase in the concentration of DOPAC [2,4]. From these findings the investigators suggested [4] and later confirmed [2] that DOPAC is not a simple alternative to the formation of HVA. Such a conclusion can now be supported by the fact that DOPAC can be reduced to DOPET.

Although DOPAC reductase was sampled initially from the arcuate nucleus, we have established its presence in the substantia nigra and in the CSF (sampled intracerebroventricularly). Similarly, DOPET was detected in these three sites; its concentration in the arcuate nucleus, substantia nigra and CSF was found to be 110 ± 12, 215 ± 25 and 60 ± 7 nM, respectively. The low level of extracellular DOPET could be due to its rapid distribution into target tissues. These data indicate that DOPAC reductase is probably subserving a global role in the brain. As the level of DOPAC in dopaminergic nuclei is in the micromolar range (1.81 μM for striatum [5], 2-3 μM for nucleus accumbens [6]), a K_m of 32 μM for DOPAC is also indicative of the physiological role of the enzyme. This role, however, appears to be a specific one as the equivalent acid metabolite of noradrenaline (DOMA), and the methoxylated acids of both noradrenaline and dopamine (VMA and HVA) were found not to be substrates of DOPAC reductase. Thus, DOPAC reductase may be an enzyme that is specifically involved in the one-step conversion of DOPAC to DOPET in the central metabolism of dopamine.

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